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Identification of the Binding Region of Basic Calponin on α and β Tubulins[1]

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Calponin is a basic smooth muscle protein capable of binding to actin, calmodulin, tropomyosin, and phospholipids. We have found that the basic calponin interacted with brain tubulin under polymerized and unpolymerized conditions *in vitro* [Fujii, T., Hiromori, T., Hamamoto, M., and Suzuki, T. (1997) *J. Biochem.* 122, 344-351]. We examined the calponin-binding site on the tubulin molecule by sedimentation, limited digestion, chemical-cross linking, immunoblotting, and delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometric (DE MALDI-TOF) analyses. Calponin interacts with both the α and β tubulins and only slightly with the tyrosinated and acetylated form of α tubulin. The binding of calponin to microtubules was blocked by adding poly(L-aspartic acid) (PLAA) or MAP2. After digestion of microtubule proteins with subtilisin, the amount of calponin binding to αβs microtubules was reduced compared to native microtubules, but no further reduction was observed in the case of αsβs microtubules. The chemical cross-linked products of calponin and synthesized peptides (KDYEEVGVDSVEGE; α-KE) derived from the C-terminal region of α tubulin and (YQQYQDATADEQG; β-YG) and (GEFEEEGEEDEA; β-GA) from that of β tubulin were detected by mass spectrometry. One kind of calponin-peptide complex was formed in the presence of α-KE or β-YG, while five complexes (calponin:peptide=1:1-5) were generated in the presence of β-GA. Peptides α-KE and β-GA inhibited the binding of calponin to tubulin produced by EDC in a concentration-dependent manner. These findings suggest that basic calponin interacts with both tubulin subunits and that their C-terminal regions, which also contain the binding sites of MAP2, tau, and kinesin, may be involved in calponin-binding.

Key words: calponin, chemical cross-linking, interaction, MALDI-TOF/MS, tubulin.

Calponin was originally discovered in smooth muscle as an actin-, calmodulin-, and tropomyosin-binding protein (*1*). Recently, three types of the calponin isoforms, acidic (pI=5-6), neutral (pI=7-8), and basic (pI=8-10), have been classified on the basis of their isoelectric point (*2-6*). Basic calponin is distributed relatively specifically in smooth muscle tissues. The binding of basic calponin to F-actin causes an inhibitory effect on actomyosin ATPase activity (*7-11*) and on the movement of actin filaments over myosin filaments *in vitro* (*12, 13*). The binding of calponin to F-actin is modulated by calmodulin and S100 in a Ca^{2+}-dependent manner (*7, 9-11*) and by its phosphorylation and dephosphorylation (*8, 14-16*). Recently, calponin has been proposed to interact with myosin (*17*), S100 (*18, 19*), phospholipids (*20*), caldesmon (*21*), desmin (*22, 23*), and microtubules (*24*).

Cytoplasmic microtubules are representative cytoskeletal elements found in almost all eukaryotic cells like microfilaments and intermediate filaments. Tubulin, a main component of microtubule proteins, is known to interact with MAPs including structural MAPs (MAP1A, MAP1B, MAP2A, MAP2B, MAP2C, and tau) and motor MAPs (MAP1C and kinesin) (*25, 26*). The structural MAPs lead to the promotion of microtubule assembly and stabilization of microtubules, and may be involved in the formation and maintenance of neuronal cells. Also, motor MAPs may be responsible for organelle transport involved in microtubule-based movement, because they can produce force through their binding to microtubules. Tubulin consists of two non-identical polypeptides designated α and β. Both subunits are subject to several post-translational modifications, such as phosphorylation (α, β), tyrosination (α), acetylation (α), and glutamylation (α, β) (*27-30*). Most of these modifications occur at or near the carboxyl termini of both subunits, which are remarkably rich in acidic amino acids, especially glutamic acid (*28, 30-32*). The carboxyl terminal regions are believed to be involved in the tubulin binding to MAPs. MAP2 and MAP1C (brain dynein) compete for the binding to microtubules, indicating

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Abbreviations: DE MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; DTT, dithiothreitol; EDC, 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide; MAP, microtubule-associated protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; PLAA, poly(L-aspartic acid); SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

that they share a common or overlapping binding site on the C-termini of tubulin subunits (*34, 35*). Both MAP2 and tau have been shown to interact with not only tubulin but also actin, and their bindings are modulated by Ca^{2+} and calmodulin (*36, 37*). Little is known about the interconnection between the microfilaments and microtubules in smooth muscle tissues. We have recently reported that basic calponin from chicken gizzard can bind to microtubules as well as F-actin, indicating that calponin may be a microtubule-associated protein in smooth muscle (*24*). These observations have prompted us to examine a calponin-binding species of tubulin and the binding region on the tubulin molecule in detail.

In this paper, we report that calponin interacts with both α and β tubulins and that the binding region is located at the carboxyl termini on the tubulin molecule.

MATERIALS AND METHODS

Materials—Taxol was obtained as a gift from Dr. J. Johnson (National Cancer Institute, NIH). Anti-α tubulin (DM 1A) and anti-β tubulin (DM 1B) were purchased from Amersham. Anti-βI+βII tubulin (JDR.3B8), anti-βIII tubulin (SDL.3D10), anti-tyrosine α tubulin (TUB-1A2), anti-acetylated α tubulin (6-11B-1), 20 kDa poly(L-aspartic acid) (PLAA), and subtilisin Carlsberg were from Sigma. MES was from Boehringer. Peptide α-KE, β-YG, and β-GA were from Sawady Technology. EDC was from Wako. All other chemicals were of high quality commercial grade.

Preparation of Proteins—Calponin was purified from chicken gizzard according to Fujii (*38*). Microtubule proteins were prepared from rat and porcine brains by two cycles of temperature-dependent assembly-disassembly purification as previously described (*39*). Purified tubulin was further isolated from depolymerized microtubule proteins by phosphocellulose (Whatman P11) chromatography (*39*). Microtubule proteins and tubulin were dissolved in 100 mM MES-NaOH (pH 6.6), 0.5 mM $Mg(CH_3COO)_2$, and 1 mM EGTA (buffer A) containing 25% glycerol. MAP2 was purified from heat-stable MAPs by Ultrogel A6 (IBF Biotechnics) column chromatography (*40*). The peak fractions were dialyzed against buffer A containing 2 μg/ml pepstatin and stored at −80°C until use. Protein concentrations were determined according to Bradford (*41*) using bovine serum albumin as the standard or by spectrophotometric measurements using A_{280} of 6.8 for calponin, A_{280} of 11.5 for tubulin, and A_{280} of 3.3 for MAP2.

Proteolysis of Tubulin with Subtilisin—Brain microtubule proteins (2 mg/ml) which had been incubated with 20 μM taxol and 1 mM GTP at 37°C for 20 min were mixed with subtilisin (1:200, w/w) at 37°C for 30 min ($\alpha\beta$s) and 13 h (αsβs). After the addition of 5 mM PMSF, the samples were further incubated at 30°C for 30 min to completely stop the reaction, then used for the sedimentation and chemical cross-linking assays. The proteolyzed microtubule preparation was subjected to analysis by 10% SDS-PAGE at pH 9.2 to obtain optimal resolution of α and β tubulins.

Chemical Cross-Linking—A mixture of calponin (7 μM) and tubulin (2 μM) was incubated at 30°C for 5 min in 40 mM MES-NaOH (pH 6.8), 20 mM NaCl, 0.1 mM DTT, 0.15 mM $Mg(CH_3COO)_2$, 0.3 mM EGTA, and 10% glycerol, then the zero-length cross-linker, 1-ethyl-3[3-(dimethylamino)propyl]carbodiimide (EDC), was added to a final concentration of 3 mM (*24*). After 20 min of incubation, the reaction was terminated by the addition of one-fifth volume of a 5×electrophoresis sample buffer (155 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, 0.75% β-mercaptoethanol, and 0.0025% pyronin Y), then heated to 95°C for 2 min. Samples were analyzed by SDS-PAGE.

Sedimentation Assay—Cosedimentation experiments were performed as previously described (*24*). Calponin was mixed with microtubule proteins ($\alpha\beta$, $\alpha\beta$s, and αsβs) or tubulin and MAP2 in a volume of 100 μl of 60 mM MES-NaOH (pH 6.8), 0.2 mM $Mg(CH_3COO)_2$, 0.1 mM DTT, 0.4 mM EGTA, 25 mM NaCl, and 10% glycerol. The assembly of microtubules was initiated at 37°C by adding 20 μM taxol and 1 mM GTP. After 30 min of incubation, the samples were centrifuged at 100,000×g for 30 min at 25°C. The obtained supernatants and pellets were analyzed by SDS-PAGE. The relative values of calponin, tubulin, MAP1, and MAP2 were determined by densitometry.

DE MALDI-TOF Mass Spectrometry—Calponin with or without peptides treated with EDC was subjected to DE MALDI-TOF mass spectrometric analysis. Sample solution (2 μl) containing calponin (10-20 pmol) and 2 μl of matrix solution (10 mg of sinapinic acid in 1 ml of 3:7 mixture of acetonitrile/water containing 0.1% trifluoroacetic acid) in a microcentrifuge tube were mixed vigorously and centrifuged on a microcentrifuge for 1 min. One microliter of the supernatant was loaded on a sample plate with 100 sample positions. The plate was loaded into a Voyager™ Elite XL (6.6 m flight length) Biospectrometry™ Workstation (PerSeptive Biosystems) with an N_2 laser (337 nm) in the linear mode. The resolution of the ion peak was determined by the resolution calculator from the GRAMS/386 software supplied with the instrument. The mass spectra were smoothed by 5-point Savitsky-Golay smoothing.

SDS-PAGE and Immunoblotting—SDS-PAGE was performed using the method of Laemmli (*42*). To improve the separation of α and β subunits of tubulin, we employed 10% polyacrylamide gel (acrylamide/bisacrylamide=200:1, w/w) containing 0.1% SDS (Sigma) at pH 9.2 (*43*). For immunoblotting, proteins were electrophoretically transferred from the gel to nitrocellulose (*44*). The sheet was blocked with 5% bovine serum albumin and 1% calf serum in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (TBS) and incubated for 1 h with antisera to calponin or tubulin, then treated with either peroxidase-conjugated goat anti-mouse or rabbit IgG for 1 h. After the immunoreaction, the sheet was washed with TBS containing 0.1% Triton X-100. The immunoreactive bands were visualized using 4-chloro-1-naphthol and H_2O_2 as the substrate for peroxidase.

RESULTS AND DISCUSSION

Interaction of Calponin with Brain Tubulin Subunits—EDC, a water-soluble zero-length cross-linking reagent, is useful for identifying proteins that closely interact with each other. As previously reported (*24*), a 86 kDa product was generated when calponin and tubulin were mixed with EDC and subjected to SDS-PAGE and Coomassie Blue

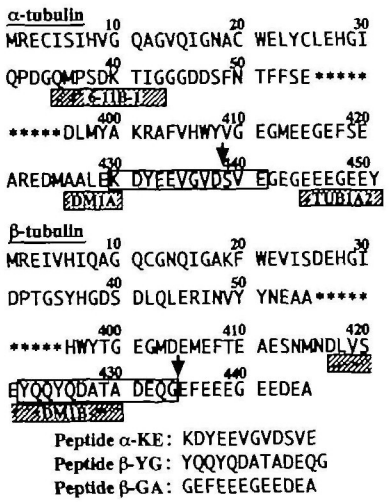

Fig. 1. **Schematic illustration of epitope locations, cleavage sites, and binding sites for MAP2 on C-terminal sequence of porcine α and β tubulins.** Amino acid sequences of porcine α and β tubulins are shown (*31, 32, 45*). Epitope locations of DM1A, DM1B, TUB-1A2, and 6-11B-1 are shown by hatched boxes (*27, 48*). Arrows indicate the positions of subtilisin cleavage sites (*28, 29, 46*). The binding sites of the 18–22 amino acid repeats from MAP2 and tau are indicated in the sequence as boxed areas (*52, 53*). The sequences of the synthetic peptides used in this study are also shown.

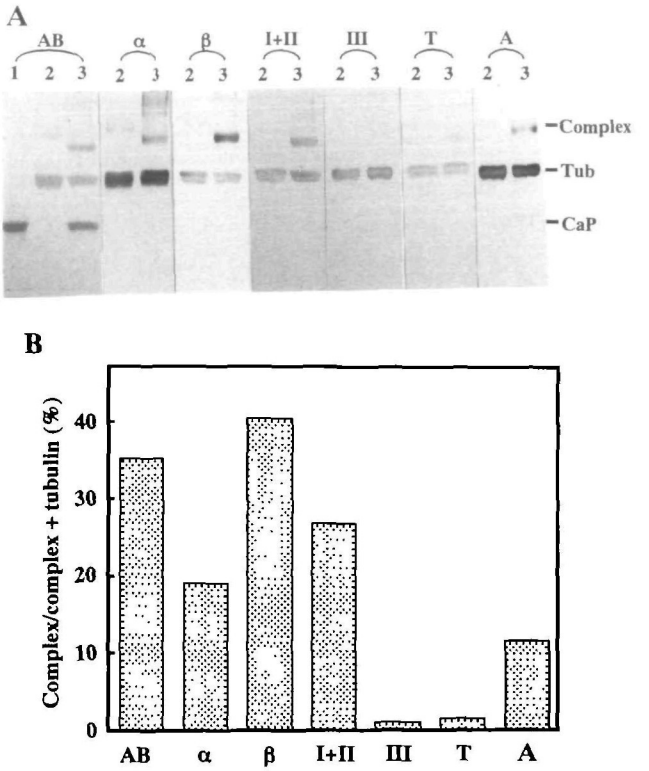

Fig. 2. **EDC cross-linking of calponin and tubulin and immunological analysis of the 86 kDa product.** (A) The cross-linking reaction was performed with 3 mM EDC for 20 min at 30°C in the solution containing 1.9 μM tubulin and 5.7 μM calponin as described in "MATERIALS AND METHODS." The cross-linking materials were separated by 10% SDS-PAGE. Proteins were electrophoretically transferred from the gel to nitrocellulose, then stained with amide black or immunostained with anti-tubulin antibodies. 1, Calponin (CaP); 2, tubulin (Tub); 3, calponin+tubulin. AB, amide black stainin; α, anti-α tubulin (DM 1A); β, anti-β tubulin (DM 1B); I+II, anti-βI+βII tubulin (JDR.3B8); III, anti-βIII tubulin (SDL.3D10); T, anti-tyrosine α tubulin (TUB-1A2); A, anti-acetylated α tubulin (6-11B-1). (B) The ratio of the 86 kDa product to total tubulin immunostained by the antibodies was estimated by scanning the sheets.

staining, indicating that the product is a 1:1 molar covalent complex of calponin (33 kDa) and tubulin subunit (50–55 kDa). We further examined the tubulin composition of the 86 kDa product. Six antibodies were used, the epitope localization of four of which are shown in Fig. 1. Immunological analysis with subunit-specific antibodies was used to confirm the composition of the 86 kDa product (Fig. 2). Both DM1A (α tubulin) and DM1B (β tubulin) antibodies cross-reacted with the 86 kDa band as well as 50–55 kDa tubulin subunits. The reactivity of DM1B with the 86 kDa product was about twofold higher than that of DM1A, indicating that β tubulin preferentially binds to calponin compared with α tubulin. At least six or seven distinct classes of α and β tubulins have been found in mammalian cells (*34, 35*). JDR.3B8, which recognizes βI+βII tubulin isoforms, reacted with both the 86 kDa and tubulin bands, while SDL.3D10, which recognizes βIII tubulin, reacted only with the tubulin band. The reactivity of the 86 kDa band with 6-11B-1 (anti-acetylated α tubulin) was about half that with DM1A, and the 86 kDa band was only slightly detected by TUB-1A2 (anti-tyrosine α tubulin). These results suggest that the isoforms of tubulin might be involved in a functional heterogeneity upon its binding to calponin.

Effects of PLAA and MAP2 on the Interaction of Calponin with Microtubules—We previously reported that acidic polyamino acids including PLAA can potentially inhibit microtubule assembly by binding to MAP1 and MAP2 (*40*). To analyze the possible association of PLAA with calponin, we performed cosedimentation experiments (Fig. 3, A–C). Like MAP1 and MAP2, calponin cosedimented with taxol-polymerized microtubule pellets after centrifugation at 100,000×*g* for 30 min at 25°C, under which conditions only a small amount of calponin itself was recovered in the pellets. The addition of 20 kDa PLAA (30 μg/ml) caused the dissociation of 20–25% of calponin from microtubule-bound calponin, while about 90% of MAP1 and 50% of MAP2 were released and recovered in the supernatant. Higher concentrations of PLAA were required for the release of calponin and MAP2. When calponin was applied to PLAA-Sepharose, it was retained on the column and eluted with 350 to 500 mM NaCl. These results suggest that PLAA can directly interact with calponin like MAP1 and MAP2, and calponin binding to microtubules resembles MAP2 binding to microtubules in its PLAA sensitivity. MAP2 is a heat-stable and well-characterized microtubule-associated protein. Preliminary experiments suggest that the ratio of calponin bound to microtubules without MAPs is higher than that bound to microtubules with MAPs (data not shown). We next examined the effect of MAP2 on the interaction between calponin and taxol-polymerized tubulin (Fig. 3D). The amount of calponin bound to microtubules decreased with increasing concentration of MAP2. The inhibition of calponin-tubulin interaction was also observed using the EDC assay. Two possibile explanations can be considered for this inhibition: MAP2 and calponin may bind

to a common region or overlapping regions of tubulin; or the binding of MAP2 may give rise to steric hindrance.

Binding of Calponin to Subtilisin-Cleaved Microtubules—Subtilisin, a microbial serine protease, produces a distinct cleavage pattern at the carboxyl terminal of both α and β tubulins, and the main product, which carries a reduced negative charge, retains the ability to form microtubules as $\alpha\beta$s and αsβs microtubules (Fig. 1) (*46, 47*). Thus, subtilisin treatment is a simple and convenient procedure to determine the importance of the C-terminal of tubulin in association with MAP2, tau, kinesin, and dynein (*34, 35, 46, 48, 49*). Replacement of low grade SDS with electrophoresis grade SDS and electrophoresis with high pH running buffer afforded a better separation of α and β tubulin subunits as individual bands (*43*). When microtubule proteins were digested with 1% (w/w) subtilisin for 30 min at 30°C, $\alpha\beta$s-tubulin was formed immediately, whereas about 13 h of incubation was required to obtain αsβs tubulin under our experimental conditions. These two forms of microtubules ($\alpha\beta$s and αsβs),which polymerized in the presence of 10 μM taxol and 1 mM GTP, were compared with native microtubules with respect to their binding to calponin using cosedimentation experiments (Fig. 4). When calponin was incubated with $\alpha\beta$s microtubules, the amount of calponin bound to the microtubules decreased to 60% of the control level. Further digestion of $\alpha\beta$s with subtilisin, to form αsβs microtubules did not affect the binding ratio, indicating that the C-terminal domain of the β subunit is related to the binding capacity of microtubules with calponin, but that of α subunit may not be responsible for binding to calponin.

Interaction of Calponin with Peptides Derived from the C Termini of α and β Tubulins—MAP2, tau, and MAP4 have been shown to contain a homologous microtubule-binding domain, the assembly-promoting (AP) sequence, which is located in their C-terminal domains and is comprised of three to four imperfect repeats of an 18-22 amino acid residue motif (*50-52*). The repeats of the AP sequence are considered to be essential for the promotion of microtubule assembly through their interaction with the C-terminal acidic portion of tubulin subunits. The amino acid sequences Lys^{430}-Glu^{441} of α tubulin and Tyr^{422}-Gly^{434} of β tubulin, which are conserved in tubulin isoforms, are considered to be crucial for the binding of these MAPs (Fig.

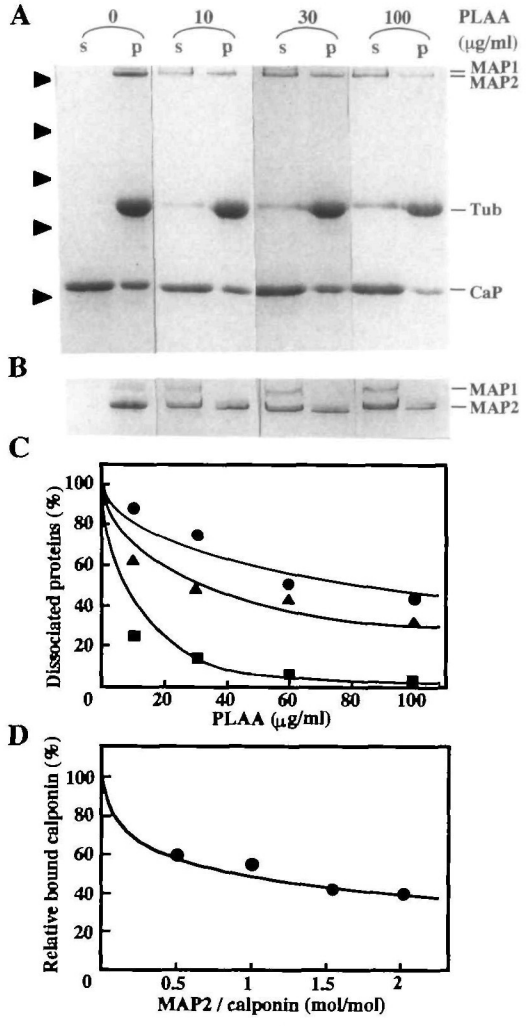

Fig. 3. **Effects of PLAA and MAP2 on the binding of MAP1, MAP2, and calponin to microtubules.** (A, C) Sedimentation assays were performed as described in "MATERIALS AND METHODS," in media containing 5.4 μM calponin, 0.38 mg/ml microtubule proteins, and various concentrations of PLAA as indicated (A) or 1.4 μM calponin, 0.8 μM tubulin and various concentrations of MAP2 as indicated (C). Samples were also analyzed by 5% SDS/urea-PAGE (B). PLAA was not stained with Coomassie Brilliant Blue R as previously described (*37*). The arrowheads indicate molecular-mass standards with the following masses: 200, 94, 67, 43, and 30 kDa. CaP, calponin; Tub, tubulin. (B, D) The amounts of calponin (●), MAP1 (■), and MAP2 (▲) were determined by densitometry.

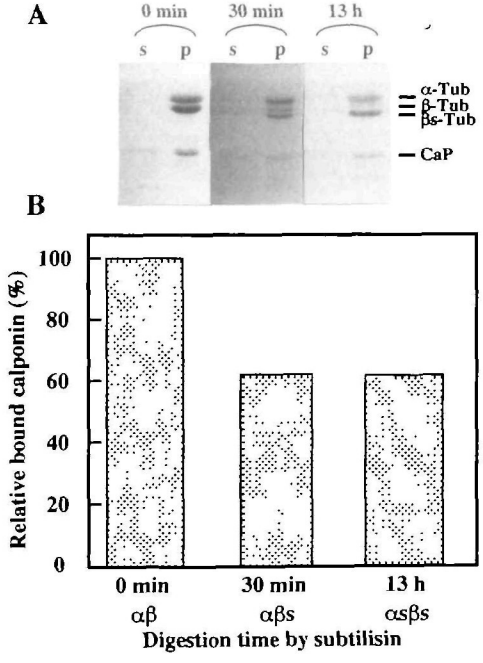

Fig. 4. **Interaction of calponin with $\alpha\beta$-, $\alpha\beta$s-, or αsβs-microtubules.** Microtubule proteins, which were incubated with 20 μM taxol and 1 mM GTP at 37°C for 20 min, were digested with 1/200 subtilisin (w/w) for 30 min ($\alpha\beta$s) and 13 h (αsβs) at 37°C. The digestion was stopped by the addition of 5 mM PMSF. The assay conditions were the same as those in Fig. 3A. The concentrations of calponin and microtubule proteins were 6.5 μM and 0.5 mg/ml, respectively.

1) (*33, 45, 53, 54*). Calponin might bind to α and β tubulin subunits *via* their homologous binding sites to the MAPs. In addition, the data of limited digestion with subtilisin suggested that the C-terminal fragment of β tubulin takes part in calponin binding to microtubules (Fig. 4). To examine these possibilities, we prepared three synthetic peptides corresponding to the C-terminal domains of α and β tubulins (Fig. 1). The calponin treated with EDC was directly analyzed by DE MALDI-TOF mass spectrometry, because the EDC cross-linking products of calponin and the peptides were not detected on SDS-PAGE. The molecular mass observed for the major species was 32,565 Da (Fig. 5A) which is in almost perfect agreement with the value of 32,333 Da calculated from the deduced amino acid sequence for chicken gizzard calponin (*2*). The molecular masses of the peptides α-KE (KDYEEVGVDSVE; molecular mass = 1,368), β-YG (YQQYQDATADEQG; molecular mass = 1,516), and β-GA (GEFEEEGEEDEA; molecular mass = 1,369) on DE-MALDI-TOF mass analyses were 1,368, 1,516, and 1,369 Da, respectively. Calponin was mixed with these peptides, then incubated with 3 mM EDC for 30 min at 30°C. The compositions of the mixtures were examined by mass spectrometry (Fig. 5, B-D). In addition to the calponin peak, we observed the presence of new peaks with molecular masses of 33,968 Da (α-KE-calponin) and 34,182 Da (β-YG-calponin), corresponding 1:1 (mol:mol) cross-linked products of α-KE-calponin (33,683) and β-YG-calponin (33,831) from the theoretical masses showing one binding site between them (Fig. 5, B and C). When β-GA and calponin were incubated with EDC, five peaks with molecular masses of 33,916, 35,317, 36,641, 38,029, and 39,356 Da were observed under the described conditions (Fig. 5D). These correspond to the calponin-β-GA, -$(\beta\text{-GA})_2$, -$(\beta\text{-GA})_3$, -$(\beta\text{-GA})_4$, and -$(\beta\text{-GA})_5$ complexes based on their molecular masses, and the calponin peak almost disappeared (Fig. 5D). This means that one calponin contains several binding sites for β-GA.

Effect of C-Terminal Peptides on Calponin-Microtubules Interaction—We examined the effects of the three synthetic peptides on the calponin binding to microtubules using chemical cross-linking and cosedimentation experiments (Fig. 6). As shown in Fig. 2, calponin was cross-linked to tubulin by EDC. The addition of α-KE or β-GA inhibited the production of the EDC complex in a concentration-dependent manner, while β-YG had little effect (Fig. 6A). The inhibitory action of β-GA was stronger than that of α-KE, because of the higher amount of β-GA bound to calponin, as shown in Fig. 5. The synthetic peptides

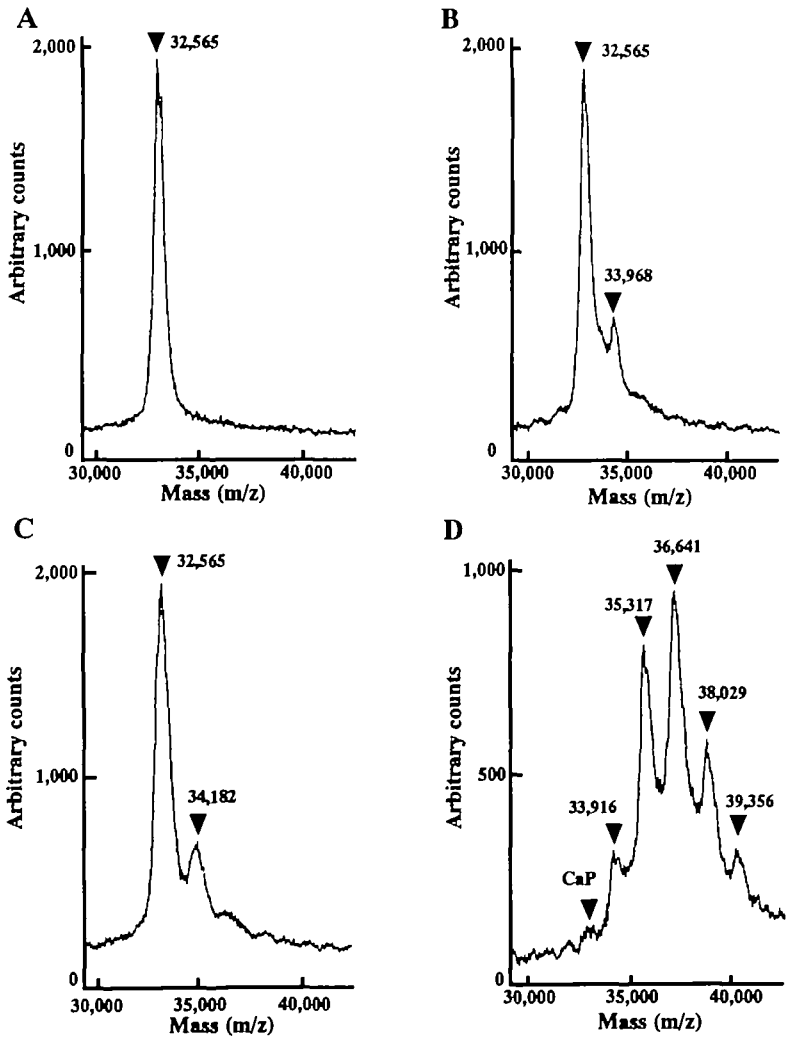

Fig. 5. **Analysis of cross-linked products of calponin and synthesized peptides using DE MALDI-TOF mass spectrometry.** The synthetic peptides of α-KE (KDYEEVGVDSVEGE), β-YG (YQQYQDATADEQG), and β-GA (GEFEEEGEEDEA) derived from α and β tubulins (see Fig. 1) were cross-linked with EDC to calponin (7.3 μM) as shown in Fig. 2. The samples were analyzed in the DE MAODI-TOF mass spectrometer. Accelerating voltage, 21,000 V; grid voltage, 85% of the accelerating voltage; grid wire voltage, 0.2% of the accelerating voltage; delay, 100 ns; laser step, 3,000; scan average, 100. Myoglobin (molecular mass = 16,950.7) and angiotensin I (molecular mass = 1,296.7) were used as external standards to calibrate the spectra.

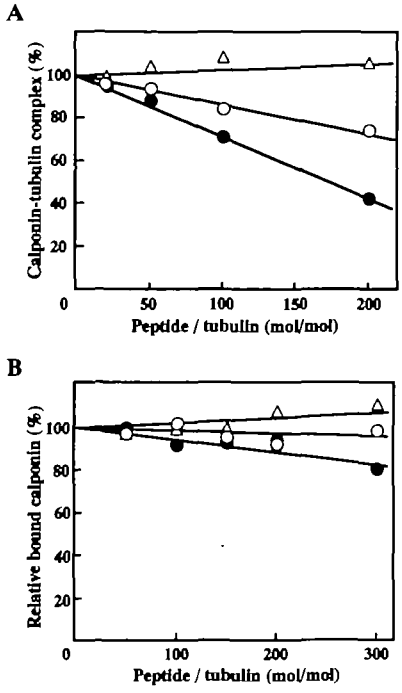

Fig. 6. **Effects of α-KE, β-YG, and β-GA on calponin-tubulin interaction by chemical cross-linking and sedimentation experiments.** (A) Calponin (7.3 μM) was cross-linked with EDC to tubulin (2.2 μM) in the presence of α-KE (○), β-YG (△), and β-GA (●) as shown in Fig. 2. (B) The assay procedure for the cosedimentation assays was the same as shown in Fig. 3. The reaction mixture contained 5.4 μM calponin, 2.7 μM tubulin, and various concentrations of the peptides as indicated. The amounts of the 86 kDa product (A) and calponin cosedimented with microtubules (B) were determined by densitometry.

failed to alter significantly the amount of calponin bound to microtubules by sedimentation assay, even when the molar ratio of peptide to tubulin was 300 (Fig. 6B). Furthermore, the mixtures of α-KE and β-YG, α-KE and β-GA, and α-KE, β-YG, and β-GA also had little influence on the calponin binding to microtubules. These findings indicate that the C-terminal regions of tubulin subunits are capable of binding to calponin, and another portion of tubulin will be necessary to block the calponin binding to microtubules.

This study has found that calponin binds to both α and β tubulins, and that the binding is dependent on tubulin isoforms and post-translational modifications. Like MAP2 and tau, calponin can bind to the synthetic peptides derived from the acidic regions of α and β tubulins. The isoelectric point of chicken gizzard calponin (pI=9.9) is similar to the calculated value of the AP sequence (pI=7.8-11.5) from MAP2 and tau (*2, 3*). However, no extensive homologous amino acid sequence was found between the AP sequences and calponin. The results from the synthetic peptides and limited digestion with subtilisin suggest the possibility that tubulin may have multiple binding sites for calponin. When tubulin was incubated with EDC for over 30 min, a tubulin-tubulin dimer product (100-110 kDa bands on SDS-PAGE) was observed, as previously reported (*55-57*), and the yield of the product was substantially reduced in the presence of calponin (data not shown). The intradimer bound between α and β tubulins detected by the EDC cross-linking reaction is formed by the N-terminal portion of α tubulin and the C-terminal portion β tubulin (*56, 57*). These data demonstrate the possibility that calponin may also bind to the N-terminal region of α tubulin. It should be noted that tau binds to 1-75 peptides from α tubulin as well as the C-terminal region of α and β tubulins in blot overlay assays (*58*). Recently, we have found that calponin can cross-link microtubules and induces the bundling of microtubules (manuscript in preparation). In fact, the interface of calponin involved multiple contact sites for β-GA (Fig. 5). Also, the tubulin-binding site on the calponin molecule remains to be elucidated. This investigation is currently in progress.

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